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The C. elegans Gene *lin-44*, Which Controls the Polarity of Certain Asymmetric Cell Divisions, Encodes a Wnt Protein and Acts Cell Nonautonomously

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Summary

Mutations in the C. elegans gene *lin-44* lead to reversals in the polarity of certain asymmetric cell divisions. We have discovered that *lin-44* is a member of the *Wnt* family of genes, which encode secretory glycoproteins implicated in intercellular signaling. Both in situ hybridization experiments using *lin-44* transcripts and experiments using reporter constructs designed to mimic patterns of *lin-44* expression indicate that *lin-44* is expressed in hypodermal cells at the tip of the tail and posterior to the cells with polarities affected by *lin-44* mutations. Our mosaic analysis indicates that *lin-44* acts cell nonautonomously. We propose that LIN-44 protein is secreted by tail hypodermal cells and affects the polarity of asymmetric cell divisions that occur more anteriorly in the tail.

Introduction

Many cell divisions during development generate two daughter cells that have distinct fates. Such divisions are said to be asymmetric (Horvitz and Herskowitz, 1992). In the nematode Caenorhabditis elegans, an important feature of most asymmetric cell divisions is that they occur with a defined orientation with respect to the body axes. The relative orientation of the different daughter cells to each other and to the body axes gives each asymmetric cell division a polarity. Very little is known about how these polarities are established. Loss-of-function mutations in the C. elegans gene *lin-44* are unusual in that they affect the polarities of certain asymmetric cell divisions while maintaining the asymmetries and orientations of division planes; thus, in *lin-44* mutants, the polarities of certain asymmetric cell divisions are frequently reversed (Herman and Horvitz, 1994). The cells with polarities affected by *lin-44* mutations are related by their positions in the tail of the animal and not by lineage history.

We have cloned *lin-44* and found that it is a member of the *Wnt* gene family. *Wnt* genes encode a large family of signaling proteins implicated in many developmental processes (reviewed by Nusse and Varmus, 1992). We show that *lin-44* is not required in the cells with polarities affected by *lin-44* mutation but rather appears to function in the tail hypodermal cells. These results suggest a model in which LIN-44 protein is a signal that is secreted by tail hypodermal cells and controls the polarity of asymmetric cell divisions occurring more anteriorly in the tail of the animal.

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Results

Positional Cloning of *lin-44*

The deficiency *hDf7* removes the *lin-44* locus and markers on each side of it (Herman and Horvitz, 1994). We located the endpoints of *hDf7* on the physical map of the C. elegans genome (Coulson et al., 1991) by using mapped cosmids as probes on genomic Southern blots of wild-type and deficiency DNA. We found that the left endpoint of *hDf7* lies in the cosmid C24G7 and that the right endpoint lies in the cosmid C47D10 (data not shown; Figure 1A). We further localized *lin-44* by generating transgenic lines of candidate genomic clones through germline transformation (Mello et al., 1991) and testing the lines for their abilities to rescue the phasmid dye-filling defect caused by *lin-44* mutation (Herman and Horvitz, 1994). In some cases, the structure of the male tail, which is grossly aberrant in *lin-44* males, was also scored for rescue.

The 350 kb YAC clone Y48F5, which spans the *hDf7* interval, rescued the *lin-44* phenotype (Figure 1A). However, cosmids that had been mapped to the interval did not rescue, which suggested that *lin-44* lies in one of the two regions of Y48F5 not covered by cosmids. A smaller derivative of Y48F5 specific to one of these gaps, SP#9, rescued the *lin-44* phenotype (Figure 1A). To narrow the rescuing region further, we used end sequences from cosmids near the ends of SP#9 to identify two overlapping genomic phage clones, which rescued the *lin-44* phenotype (Figure 1B). The rescuing region was further delimited to 4.1 kb (Figure 1B).

A 1.2 kb cDNA Corresponds to the *lin-44* Locus

We determined the sequences of both strands of a cDNA specific to the 4.1 kb genomic region and one strand of the 4.1 kb genomic clone. This analysis revealed the gene structure shown in Figure 1C. The presumptive *lin-44* cDNA is 1278 bp long, contains seven exons, and is derived from a 3267 bp segment of genomic DNA. The complete sequence of the *lin-44* cDNA is shown in Figure 2. The cDNA contains an open reading frame that could encode a protein of 348 amino acid residues, a 200 nt 3′ untranslated region that includes the polyadenylation signal AATAAA, and a poly(A) tail. Northern blot analysis using the cDNA clone as probe revealed a message of approximately 1.2 kb (data not shown). The genomic sequence contains an in-frame stop codon 12 nt upstream of the putative translational start site with no consensus 3′ splice acceptor sequence between the stop codon and

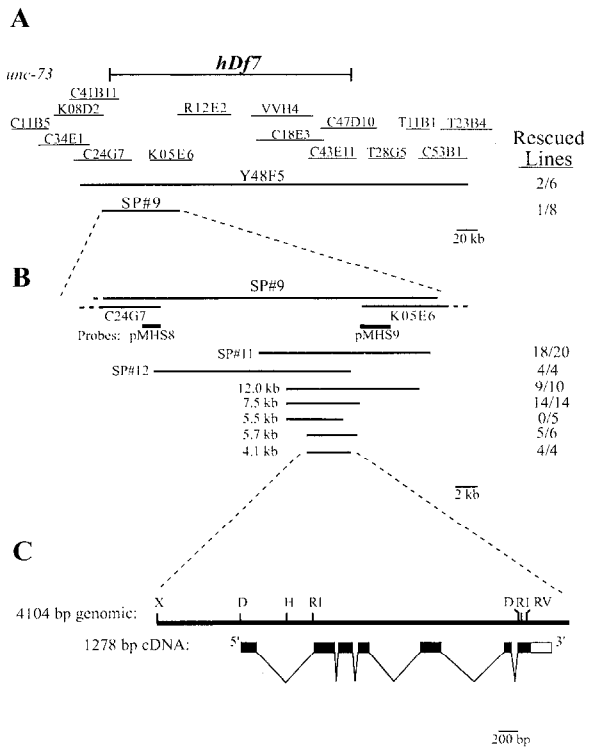

Figure 1. Positional Cloning of the *lin-44* Locus

(A) Physical map of overlapping cosmids and YAC Y48F5 in the region of *hDf7*. None of the cosmids in the *hDf7* interval was found to rescue a *lin-44* mutant in microinjection experiments (data not shown). Two lines of *lin-44* animals transgenic for the YAC clone Y48F5 were rescued. The number of lines rescued for the phasmid defect per total number of transgenic roller lines generated is given. Y48F5 transgenic males were also rescued for the male tail defect. A derivative of Y48F5, SP#9, also rescued the *lin-44* mutant phenotype.

(B) Subclones of the SP#9 interval. Genomic λ clone SP#11 was isolated from a C. elegans genomic library using pMHS9 as a probe. pMHS9 is the 3.0 kb EcoRI–NdeI end fragment of K05E6. The λ clone SP#12 was isolated from a genomic library constructed from the yeast strain containing SP#9 using pMHS8 as a probe. pMHS8 is the 1.6 kb EcoRI end fragment of C24G7. The number of lines rescued for the phasmid defect per total number of transgenic roller lines generated is shown for these genomic clones and for subclones of SP#11. Males transgenic for the 7.5 kb subclone were also rescued for the male tail defect.

(C) Molecular map of the *lin-44* locus. Sequence analysis of the 4.1 kb genomic clone and a 1278 bp cDNA specific to this fragment revealed the intron/exon structure shown. Boxes indicate exons; closed portions indicate the open reading frame. Restriction sites shown: D, DraIII; H, HindIII; RI, EcoRI; RV, EcoRV; X, XbaI. The GenBank accession numbers are U22184 for the 4.1 kb *lin-44* genomic clone and U22179 for the *lin-44* cDNA clone.

the translational start site. Together, these data support the view that the cDNA is full length or nearly full length.

We determined the sequences of both *lin-44* mutant alleles (Figure 2). The *n1792* mutation was associated with a G-to-A transition at nucleotide 306, changing the tryptophan codon at amino acid residue 100 of the putative protein of *lin-44* to an amber stop codon. The *n1792* allele was earlier shown to be suppressed by the amber suppressor *sup-7* (Herman and Horvitz, 1994). The *n2111* allele was associated with an insertion of a T among nucleotides 58–64 of the cDNA, introducing a frameshift after the first 20

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cagcgtgATGCGAGCAGCTCCTTTTGATTTCTTTTTTCAATCGACTGCTCTTTCA
       M  R  A  A  P  F  D  F  F  F  Q  S  T  A  L  S
n2111: T
       v
ACTTTTTTTATTCTTTGCTCCCTGGCCACGAATGAGATCCCGACGATCAGTGGTG
T  F  F  I  L  C  S  L  A  T  N  E  I  P  T  I  S  G  A
                                                    ↓
CACCTGCAGGGAAGATCGTTCAACCACCTAAGCCAAATATTTTGAAACAGGGTTG
  P  A  G  K  I  V  Q  P  F  K  P  N  I  L  K  Q  G  C

CCCATCAGATCTTCTGCATTCACGTGCTCTTCGATCAATTCAACTGGCATGCCGT
 P  S  D  L  L  H  S  R  A  L  R  S  I  Q  L  A  C  R

ACACATCCTGCTACAGTAATCTCGGCGTTCGAAGGAGTTCAGGAAGGACTTCAAA
T  H  P  A  T  V  I  S  A  F  E  G  V  Q  E  G  L  Q  N
                    n1792:  A
                            ↑
ACTGTGCGAATCGTTTGAGATTTCAGCAATGGGATTGTTCGGAAGCTGGAAATAT
  C  A  N  R  L  R  F  Q  Q  W  D  C  S  E  A  G  N  I
                            ↓
TATGCATGATCCGCCGTTATTGAGACAGGGTTTCCGAGAGTCTTCTCTGATTTGG
 M  H  D  P  P  L  L  R  Q  G  F  R  E  S  S  L  I  W

GCGTTGTCATCTGCATCTGCCGCCTGGGGAGTTGCGACGGCATGCGCACAAGGAT
A  L  S  S  A  S  A  A  W  G  V  A  T  A  C  A  Q  G  W

GGATTGACGATTGTGCGTGTAACAACCAGATGGGTCAAAATGAATACGAGTTCGG
  I  D  D  C  A  C  N  N  Q  M  G  Q  N  E  Y  E  F  G
  ↓
CGGATGTACTCATGGTGTTCAACACGGAATAACGGCTAGTCGAAAGCTGCTCACA
 G  C  T  H  G  V  Q  H  G  I  T  A  S  R  K  L  L  T

AAGGTCGGAGCGGTGAATACTTTGTTAAGGAAGGTGGAGAAGCACAATTTGAAGG
K  V  G  A  V  N  T  L  L  R  K  V  E  K  H  N  L  K  A
         ↓
CAGGGAGATTGGCCATCAAAAAGACCCTAATCTCTTCCTGCAAATGCCACGGAGT
  G  R  L  A  I  K  K  T  L  I  S  S  C  K  C  H  G  V

ATCCGGTTCTTGTCAACAGAAAACTTGCTGGAAACGAACTGCAACCCTTGAGCAC
 S  G  S  C  Q  Q  K  T  C  W  K  R  T  A  T  L  E  H

ATTACCGATTATTTAGTGGAAAAGTATGCACGAGCCAAGCTCTATACGGATGATT
I  T  D  Y  L  V  E  K  Y  A  R  A  K  L  Y  T  D  D  S

CGGTGGTAAAGACGACGGATTTGATATATTTAGAAGCGTCTCCGGATGTTTGCAA
  V  V  K  T  T  D  L  I  Y  L  E  A  S  P  D  V  C  K
↓
GGCAAAATCAGTTGCCGGCAGAGTGTGTGCATGGAGGAATGAGACGCATACCCAA
 A  K  S  V  A  G  R  V  C  A  W  R  N  E  T  H  T  Q
                                   ↓
GGTGATTGTGATCGGCTGTGTTGCGGGAATGGATTCAGCATTCGTCACGAAGTGG
G  D  C  D  R  L  C  C  G  N  G  F  S  I  R  H  E  V  V

TCCGTGTGAAGTGTGACTGTGAATTCGTGTGGTGTTGCAATCTGGTCTGTAAGGA
  R  V  K  C  D  C  E  F  V  W  C  C  N  L  V  C  K  D

TTGTATCCAACATCGCTGGATATCCACGTGTAATGGGACACCGCCGAAAAGCCTA
 C  I  Q  H  R  W  I  S  T  C  N  G  T  P  P  K  S  L

ATTTTTTAAtatttaatttctttttttttgtttgatttttttcataatttccag
I  F  *

tcgaaatcagttcgttttttccatttttcctcctgcacgggtcaaccgaaactc
tctcgatttgtacaattcatcttgttttctaattacttttttgccgtccttgca
tcccacacgttttcatttcctcgcctttcttcaataaatatgctaatttatcaa

aaaaaaaaaaaa
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Figure 2. *lin-44* cDNA Sequence

The DNA sequence of the 1278 bp *lin-44* cDNA is shown above, in upper case letters for putative translated regions and in lower case letters for untranslated regions, with the conceptual translation product shown below. The putative polyadenylation signal is double underlined. The position of each intron is indicated by a down arrow. The potential signal sequence (von Heijne, 1986) is underlined, and a potential N-linked glycosylation site is shown in bold type. The nucleotide changes observed in the two *lin-44* mutants, determined in each case by determining the sequences of the exons of three independent PCR clones, are indicated above the DNA sequence.

amino acid residues of the polypeptide and a stop codon after eight more amino acid residues. Thus, changes in the cDNA sequence were detected in both *lin-44* mutant alleles, indicating that the cDNA corresponds to the *lin-44* locus.

Expression of the 1278 bp cDNA Rescues the *lin-44* Mutant Phenotype

We constructed plasmids containing the *lin-44* cDNA fused to either one of two heat shock promoters, *hsp16-2* and *hsp16-41* (Stringham et al., 1992). We constructed control plasmids by inserting 4 bp at nucleotide position 223 of the cDNA insert of each heat shock construct. This insertion results in a frameshift after the aspartic acid resi-

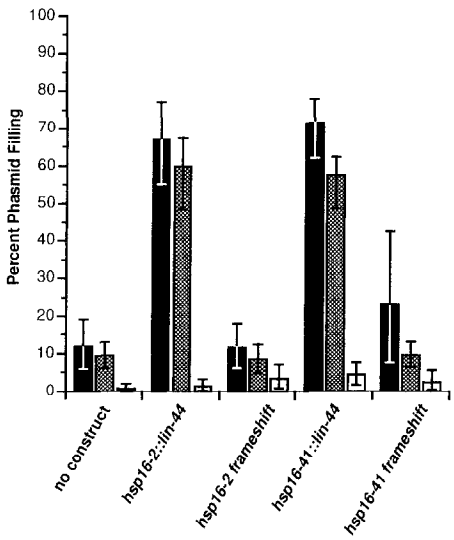

Figure 3. Expression of the *lin-44* cDNA Rescued the *lin-44* Mutant Phenotype

lin-44 animals transgenic for heat shock or control plasmids were heat shocked as embryos. Results from two independent heat shock experiments (closed and hatched bars) and a control without heat shock (open bars) are shown for each of five strains. The animals were scored for rescue of the *lin-44* phasmid-filling defect. In wild-type animals, 98% of phasmids fill with dye (Herman and Horvitz, 1994). The lanes are indicated as follows: no construct, *lin-44(n1792)*; *hsp16-2::lin-44*, *lin-44(n1792)* transgenic for the *hsp16-2* promoter fused to the *lin-44* cDNA; *hsp16-2 frameshift*, *lin-44(n1792)* transgenic for the *hsp16-2* promoter fused to the frameshifted *lin-44* cDNA; *hsp16-41::lin-44*, *lin-44(n1792)* transgenic for the *hsp16-41* promoter fused to the *lin-44* cDNA; *hsp16-41 frameshift*, *lin-44(n1792)* transgenic for the *hsp16-2* promoter fused to the frameshifted *lin-44* cDNA. Error bars are 95% confidence limits based upon binomial distributions.

due at position 56 of the putative protein. Heat shocks were performed on transgenic lines containing either one of the *hsp16::lin-44* cDNA fusion plasmids or a control plasmid. Each of the *hsp16::lin-44* cDNA fusion plasmids rescued both the phasmid (Figure 3) and male tail (data not shown) defects of *lin-44* mutants after heat shock, whereas the control plasmids did not. These data indicate that the 1.2 kb cDNA encodes a functional LIN-44 protein.

lin-44 Encodes a Member of the Wnt Family of Secretory Glycoproteins

A search of protein databases revealed that the predicted protein of *lin-44* is a member of the Wnt family of secretory glycoproteins (Figure 4). *Wnt* genes encode a large family of signaling proteins implicated in many developmental processes (reviewed by Nusse and Varmus, 1992; Siegfried and Perrimon, 1994; Klingensmith and Nusse, 1994). Comparison of the putative protein of *lin-44* to the other Wnt family members showed that LIN-44 has approximately the same similarity to DWnt-2 (33.9% sequence identity) and wingless (wg) (31.7% sequence identity) from Drosophila melanogaster and Wnt7b (31.7% sequence identity) and Wnt7a (30.3% sequence identity) from mouse. It has been proposed that Drosophila wg and DWnt-2 (an ortholog of mouse Wnt7a and Wnt7b) belong to distantly related branches of the Wnt family ancestral lineage (Sidow, 1992), and we have been unable to assign LIN-44 to any particular branch of the lineage. The predicted LIN-44 protein has several Wnt family features (Nusse and Varmus, 1992; see Figure 2). First, the 348 amino acid residues of the predicted LIN-44 protein are close in number to the average of 350–380 amino acid residues of other Wnt proteins. Second, there is a hydrophobic region at the N-terminus that could function as a signal peptide (von Heijne, 1986). Third, there is one prospective site for N-linked glycosylation near the C-terminus (other Wnt proteins contain one or two such sites). Fourth, the predicted LIN-44 protein contains 24 cysteine residues; the positions of 22 of these are strongly conserved among members of the Wnt family.

lin-44 Is Expressed in the Tail Hypodermis

We constructed a transcriptional fusion of *lacZ* (Fire et al., 1990) and a 2.6 kb genomic DNA fragment from the *lin-44* region. The 2.6 kb fragment corresponded to the genomic sequence found immediately upstream of the *lin-44* protein coding region, ending 3 nt before the translational start site. We examined animals of all ages and observed expression only in the tail. The earliest expression we detected was in three nuclei at the 1.5-fold stage (approximately 430 min) of embryonic development (Figures 5A and 5B). We observed this expression pattern in many embryos. By comparing the positions of the expressing nuclei with diagrams of nuclei in 430 min embryos (Sulston et al., 1983), we identified the expressing nuclei as hyp10, a hypodermal syncytium containing two nuclei at the tip of the tail, and hyp11, a mononucleate tail hypodermal cell. The earliest asymmetric cell divisions affected by *lin-44* mutations, those of the T cells (Herman and Horvitz, 1994), occur approximately 11 hr after the earliest detected expression in hyp10 and hyp11. Later in embryonic development, expression was observed in the other cells of the tail hypodermis, hyp8 and hyp9. In a few animals, simultaneous expression in all the tail hypodermal cells was observed (Figures 5C and 5D).

We also examined the appearance of endogenous *lin-44* transcripts by performing in situ hybridization experiments using wild-type embryos. Using an antisense *lin-44* probe, we detected hybridization only in the tail. Control experiments with a sense *lin-44* probe gave only nonlocalized background staining (data not shown). The earliest *lin-44* transcripts we detected were in the hyp10 and hyp11 cells at the 1.5-fold stage of embryogenesis (Figure 5E). We observed hybridization in the hyp10 and hyp11 cells throughout the rest of embryogenesis. At the 3-fold stage of embryogenesis, hybridization was also observed in the other cells of the tail hypodermis, hyp8 and hyp9 (Figure 5F). The only difference between this expression pattern and that seen with the *lacZ* reporter was that the in situ hybridization pattern indicated more consistent expression throughout the hyp8–hyp11 cells. Expression by the *lacZ* reporter construct was also limited to hyp8–hyp11, but many embryos showed staining of subsets of these cells, and only a small proportion showed staining of the full set of hyp8–hyp11 cells.

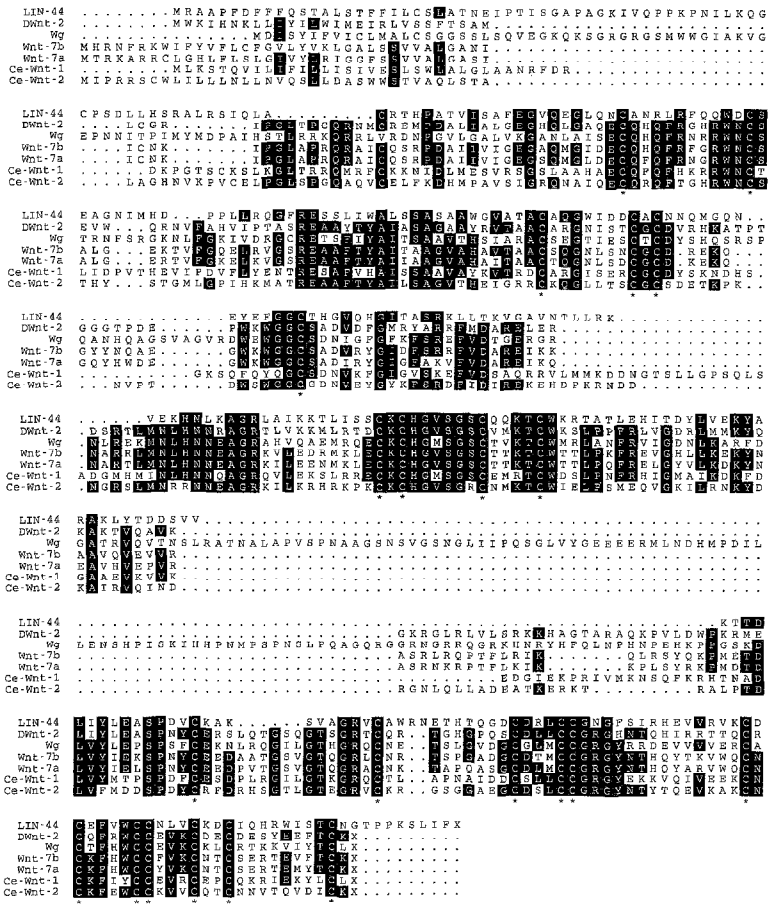

Figure 4. LIN-44 Is a Member of the Wnt Family of Secretory Glycoproteins

Alignment of LIN-44 with Drosophila DWnt-2 (Russell et al., 1992) and wg (Rijsewijk et al., 1987), mouse Wnt7a and Wnt7b (Gavin et al., 1990), and C. elegans Ce-wnt-1 and Ce-wnt-2 (Shackleford et al., 1993). Positions in which a majority of family members have identical residues are shown by black boxes. Positions of conserved cysteines are indicated by asterisks.

The in situ hybridization technique at present cannot be applied to detect expression after hatching (Seydoux and Fire, 1995), so we used the *lacZ* and green fluorescent protein (GFP) (Chalfie et al., 1994) reporter gene constructs to examine *lin-44* expression during larval development. After hatching, expression was most often observed only in hyp10 (Figure 5G). However, many animals continued to express the reporter genes in hyp8, hyp9, and hyp10 (Figure 5H) or in subsets of these tail hypodermal cells (Figure 5I). In most of the lines transgenic for either fusion construct, expression was observed, most often in hyp10, through the L4 stage, and occasionally in adult animals (data not shown). We obtained similar results with fusions using an 837 bp genomic DNA fragment that also ended 3 nt upstream of the *lin-44* coding region (Figure 5J; data not shown).

We also saw expression in the phasmid socket cells after the mid-L1 stage (when they are generated) (Figure 5J) and through the L4 stage (data not shown). We observed no other expression in the T cell lineage, and the socket cell expression was not detectable until long after *lin-44* function is needed to specify the polarity of the T cell division.

lin-44 Function Is Cell Nonautonomous

Our expression studies predicted that the focus of *lin-44* action in specifying the polarities of the T and B cells is not in the T and B cells themselves but rather in the tail hypodermis. We used mosaic analysis to test this prediction of cell nonautonomy. To generate *lin-44* mosaics, we made *lin-44; ncl-1 unc-36* animals transgenic for an extrachromosomal array of DNA, *mnEx31*, that contains wild-type copies of *lin-44*, *ncl-1*, and *unc-36*. The extrachromosomal array is subject to mitotic loss. Each loss generates a clone of mutant cells, and the *ncl-1* mutation, which results in enlarged nucleoli, is a cell autonomous marker for specifying the nature of mosaic animals (Herman, 1989; Lackner et al., 1994).

The first embryonic division produces the daughter cells AB and P_1. All of the cells with polarities affected by *lin-44* mutation descend from AB.p, the posterior daughter of AB. The tail hypodermal cells hyp8, hyp9, and hyp10 also descend from AB.p, but hyp11 descends from P_1. We first looked for hermaphrodites that had lost the array in AB or AB.p but retained it in P_1. Since the focus of *unc-36* function is among the descendants of AB.p (Kenyon, 1986), we sought such mosaics among Unc-36 animals. We screened the Unc-36 progeny of *lin-44; ncl-1 unc-36; mnEx31* hermaphrodites for animals in which cells derived from AB.p were Ncl but cells derived from P_1, particularly hyp11, were non-Ncl. We determined the cell divisions at which the array was lost in each of these animals by scoring the Ncl phenotypes of many cells, and we scored the polarities of the two T cells. Most of the Unc-36 animals

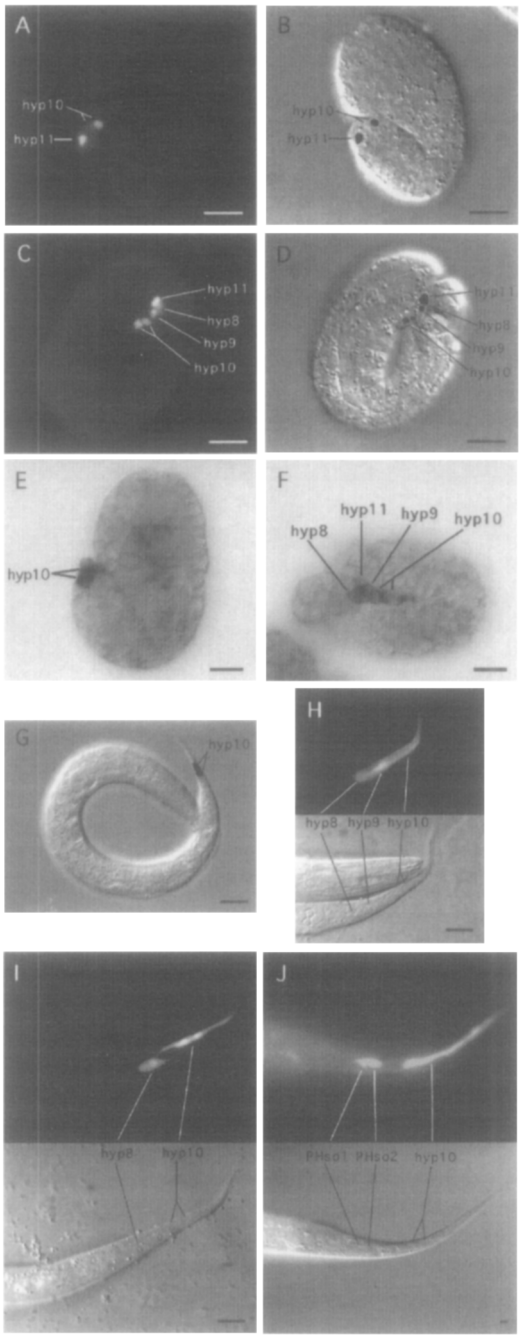

Figure 5. *lin-44* Reporter Constructs Were Expressed in the Tail Hypodermis

(A) Staining of an *unc-29; mnIs9* 1.5-fold embryo with anti-β-galactosidase antibodies. *mnIs9* is an integrated copy of a transgenic array containing a transcriptional fusion of a 2.6 kb fragment upstream of the *lin-44* coding region and the *lacZ* gene bearing a nuclear localization signal. The stained nuclei are the two hyp10 nuclei, one of which is only partially visible in this focal plane, and the hyp11 nucleus.

(B) A negative of the image in (A) overlaid onto a Nomarski micrograph of the same embryo to show the locations of the staining nuclei. The composite image was generated using Adobe Photoshop software (Mountain View, CA).

(C) Staining of an *unc-29; mnIs9* 3-fold embryo as in (A).

(D) A negative of the image in (C) overlaid onto a Nomarski micrograph of the same embryo. Part of the tail of this 3-fold embryo is twisted underneath the head below the focal plane shown; the tip of the tail reemerges into the focal plane. The stained nuclei are hyp8 (partially visible in this focal plane), hyp9, hyp10, and hyp11.

(E) In situ hybridization of a 1.5-fold embryo to antisense *lin-44* probe. The probe localized to the cytoplasm of hyp10 and hyp11, but hybridization to hyp11 was outside the focal plane of this micrograph.

(F) In situ hybridization of a 3-fold embryo to antisense *lin-44* probe. Only the focal plane containing the *lin-44* transcripts is shown. The probe localized to the cytoplasm of hyp8, hyp9, hyp10, and hyp11.

(G) X-Gal staining of an *unc-29; mnIs9* newly hatched L1 larva. Only the hyp10 nuclei stained.

(H) GFP expression in an *unc-29; mnEx45* L1 larva (upper) and the same larva as viewed with Nomarski optics (lower). *mnEx45* is a transgenic array containing a transcriptional fusion of a 2.6 kb fragment upstream of the *lin-44* coding region and the GFP gene. This construct does not contain a nuclear localization signal, so the GFP fills the cells in which it is expressed. The expressing cells were hyp8, hyp9, and hyp10.

(I) GFP expression in an *unc-29; mnEx45* L1 larva. The expressing cells in this animal were hyp8 and hyp10 only.

(J) GFP expression in an *unc-29; mnEx36* L3 larva. *mnEx36* is a transgenic array containing a transcriptional fusion of an 837 bp fragment upstream of the *lin-44* coding region and the GFP gene. The expressing cells were the left phasmid socket cells PHso1L and PHso2L and hyp10. Scale bars, 10 μm.

did not contain the array in any cells, presumably because of meiotic segregation of the array in the parent hermaphrodite. These animals served as controls: 99% (n = 647) of them had abnormal T cell polarities. We found 13 mosaic animals in which the array was absent from AB or AB.p and retained in P_1, including hyp11; we also found two animals that lost the array twice, once in the lineage leading to TL and once in the lineage leading to TR (Figure 6A). Among the total of 15 animals in which both TL and TR were genotypically mutant, 12 showed normal polarities for both T cells. We conclude that *lin-44* acts cell nonautonomously and that *lin-44* expression among one or more descendants of P_1 is usually sufficient to confer normal polarities to the T cell divisions. Three of the fifteen mosaics, however, showed abnormal T cell polarity, either for one of the T cells (one animal) or for both T cells (two animals). This result suggests that *lin-44* expression in wild-type animals may not be limited to the P_1 lineage but may also occur among descendants of AB (e.g., hyp8, hyp9, and hyp10) and that expression among descendants of P_1 alone may not always be sufficient to confer normal T cell polarity.

We also examined B cell polarity in mosaic males that had early losses in the AB.p lineage. We screened the Unc-36 progeny of *lin-44; ncl-1 unc-36; mnEx31; him-5* hermaphrodites for males in which cells derived from AB.p were Ncl but cells derived from P_1, particularly hyp11, were non-Ncl. When we found such mosaic animals, we scored the Ncl phenotype of many cells and scored the polarities of the B cell as well as of TL and TR. Most of the Unc-36 males did not contain the array; among these animals, 83% (n = 109) had abnormal B cell polarity, and 99% had abnormal T cell polarities. We found five mosaic males of the desired type, three of which had lost the array at the division that generates AB, and all five males had normal B, TL, and TR cell polarities (Figure 6B). These results indicate that *lin-44* function is not required in either the B or T cells for their polarities to be normal and that expression

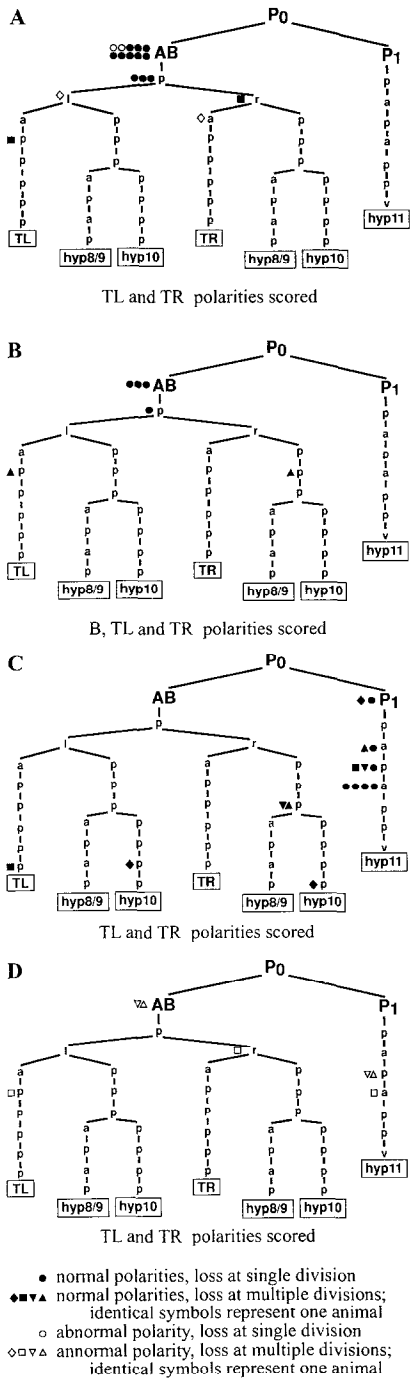

Figure 6. *lin-44* Function Is Cell Nonautonomous

Symbols indicate positions in the cell lineage at which a *lin-44(+)*-containing extrachromosomal array was lost to generate each genetic mosaic animal that was scored. The genotype of the zygotes represented in (A), (C), and (D) was *lin-44; ncl-1 unc-36; mnEx31[lin-44(+) ncl-1(+) unc-36(+)]*. The genotype of the zygotes represented in (B) was *lin-44; ncl-1 unc-36; mnEx31; him-5*. The lineages of only the relevant cells are shown. Each cell is drawn below its mother cell, and its lineage can be read directly from the diagram, where a, p, l, r, d, and v refer to anterior, posterior, left, right, dorsal, and ventral daughters, respectively. Many of the mosaic animals had additional losses of the extrachromosomal array (as determined from Ncl phenotypes of many cells) in undiagrammed parts of the lineages. Closed symbols indicate that the polarities of all the scored cells were wild type in the mosaic animal. Open symbols indicate that the polarity of at least one scored cell was abnormal.

(A) Positions in the cell lineage of array losses that generated Unc-36 hermaphrodites that were *ncl-1(+)* in hyp11. We found 15 such mosaics; 10 were AB(−) mosaics, meaning that the array was absent from cell AB but not from its sister cell P_1 (represented by circles in the diagram next to AB), three were AB.p(−) mosaics (represented by circles next to AB.p), and two animals had double losses, causing the array to be absent from both TL and TR. For one of these last two animals, the positions of the two losses are indicated by squares (at AB.plap and AB.pr), and for the other animal, the two losses are indicated by diamonds (at AB.pl and AB.pra). TL and TR cell polarities were assessed by scoring the positions of the phasmid socket cells (see Experimental Procedures). In both AB(−) mosaic animals represented by open symbols, TL and TR had abnormal polarities. In the animal represented by diamonds, the polarity of TL was wild type and the polarity of TR was abnormal.

(B) Positions in the cell lineage of array losses that generated Unc-36 males that were *ncl-1(+)* in hyp11. We found five such mosaics. Three were AB(−), one was AB.p(−), and one (represented by two triangles) had losses at both AB.plap and AB.prpp. The TL, TR, and B cells of all five males were phenotypically wild type with respect to *lin-44*.

(C) Positions in the cell lineage of array losses that generated non-Unc-36 hermaphrodites that were *ncl-1* in hyp11. For seven animals, represented by circles, a single point of array loss was found in the lineages shown. Four other animals, represented by squares, triangles, inverted triangles, and diamonds, had two or three losses. For all eleven animals, TL and TR were phenotypically wild type with respect to *lin-44*.

(D) Positions in the cell lineage of array losses that generated Unc-36 hermaphrodites that were simultaneously *ncl-1* in hyp11 and *ncl-1(+)* in some other cells descended from P_1. We found three mosaics of this type. Two, represented by triangles and inverted triangles, were AB(−) and P_1.pap(−) mosaics, and one, represented by squares, had array losses at AB.plap, AB.pr and P_1.papa. For all three animals, TL and TR were phenotypically mutant, as judged by the positions of the phasmid socket cells.

among one or more descendants of P_1 may be sufficient to confer normal B and T cell polarity.

To test our hypothesis that the action of *lin-44* is not limited to descendants of P_1, we screened non-Unc-36 animals for mosaics that had lost the array in the P_1 lineage, particularly in the cell lineage leading to hyp11. We found eleven mosaics of this type, two of which lost the array at P_1, and all had normal polarities of both TL and TR (Figure 6C). We conclude that *lin-44* action is not limited to the P_1 lineage.

The results from these mosaic experiments indicate that *lin-44* acts cell nonautonomously in specifying the T and B cell polarities and are consistent with the hypothesis that the focus of *lin-44* function is among the tail hypodermal cells hyp8–hyp10 (which descend from AB.p) and hyp11 (which descends from P_1). Because of the lineal relationships among the T cells and the hyp8–hyp10 cells, we were not able to test whether *lin-44* function in hyp8, hyp9, or hyp10 alone is sufficient to confer normal polarities to the T cells. However, we were able to test the idea that the P_1-derived cell responsible for *lin-44* action is hyp11 by identifying Unc-36 mosaics in which the extrachromosomal array was present in some descendants of P_1 but was missing in hyp11. To generate mosaics of this type, the array had to be lost at least twice, once in the AB lineage and once in P_1. We found three such mosaic

animals; the polarities of both T cells were defective in all three (Figure 6D). The only P_1 descendants missing the array in these mosaic animals were seven nuclei that are members of the large hypodermal syncytium hyp7 (which included non-Ncl nuclei), 16 body muscles, and hyp11. This result is consistent with the hypothesis that *lin-44* functions in hyp11, along with hyp8–hyp10.

Discussion

We used two independent methods for following embryonic *lin-44* expression: in situ hybridization to *lin-44* transcripts and expression of *lacZ* fused to a 2.6 kb genomic fragment from immediately upstream of the putative *lin-44* translational start site. The two methods were in excellent agreement. The earliest *lin-44* expression that we were able to detect was in the tail hypodermal cells hyp10 and hyp11 at about 430 min of embryonic development. Later in embryogenesis, all of the tail hypodermal cells hyp8–hyp11 showed *lin-44* expression, and no other cells showed detectable expression. We used both *lacZ* and a GFP gene as reporters to elucidate the pattern of postembryonic *lin-44* expression. The two reporters gave essentially identical results: hyp10 showed expression in most animals at all later stages, but we also observed expression in the other tail hypodermal cells, hyp8 and hyp9, as well as in the phasmid socket cells but in no other cells of the animal.

Our mosaic analysis indicated that *lin-44* function is not required in the T or B cells for them to exhibit normal cell polarities. The cellular focus of *lin-44* function appears to be diffuse. Our results are consistent with the idea that the focus is among the tail hypodermal cells hyp8–hyp11. *lin-44* function in one of these cells, hyp11, appears usually to be sufficient to cause mutant T or B cells to have normal polarity. Expression in tail hypodermal cells other than hyp11 also can be sufficient to promote normal polarity. Presumably normal T and B cell polarities are assured when all the tail hypodermal cells are able to express *lin-44(+)*. The observation that a single copy of the amber suppressor *sup-7(st5)* is sufficient to suppress the *lin-44(n1792)* amber mutation (Herman and Horvitz, 1994) suggests that only a small amount of *lin-44* product is required to confer normal polarity to the T and B cells, since two copies of *sup-7(st5)* restore only about 30% of normal activity to a suppressed locus (Waterston, 1981). We attempted to test whether we could mimic the effect of *lin-44* mutations on T cell polarity by using a laser microbeam to kill the tail hypodermal cells, but animals in which the hyp8–hyp11 cells were killed at the 1.5-fold stage of embryonic development did not hatch, and their tails appeared to have ruptured while elongating during morphogenesis (our unpublished results).

LIN-44 and Other Wnt Proteins Function in Cell Signaling

The LIN-44 protein appears to be a member of the Wnt family of secretory glycoproteins. LIN-44 is approximately the same size as other Wnt proteins and, like other members of the Wnt family, appears to have an N-terminal hydrophobic signal peptide, a possible site for N-linked glycosylation near the C-terminus, and a conserved positioning of 22 cysteine residues.

Two other *Wnt* genes have been identified in C. elegans, each of which encodes a protein with about 30% sequence identity to LIN-44. *Ce-wnt-1* was identified by polymerase chain reaction (PCR) using degenerate primers (Kamb et al., 1989). *lin-44* would not have been identified in that study because the sequence encoded by one of the primers is not conserved in LIN-44. *Ce-wnt-2* was identified by the C. elegans genome sequencing project (Shackleford et al., 1993). Mutations of *Ce-wnt-1* and *Ce-wnt-2* have not been reported.

The *Wnt* genes appear to encode signaling molecules that are involved primarily in short-range cell interactions. In Drosophila embryos, wg protein is found within a few cell diameters of *wg*-expressing cells (van den Heuvel et al., 1989). Evidence from mosaic analysis in Drosophila suggests that the range of wg action in maintaining expression of the *engrailed* gene is only about one cell diameter (Vincent and Lawrence, 1994). Our expression and mosaic data indicate that LIN-44 is expressed in the tail hypodermis and functions to signal the B and T cells about polarity. At the time we observed LIN-44 expression in embryos, the T cells appear to touch the tail hypodermal cells hyp10 and hyp11, as judged by staining with a monoclonal antibody that binds to adherens junctions and outlines the boundaries of hypodermal cells (Podbilewicz and White, 1994; our unpublished observations). By contrast, the B cell is several cell diameters away from the tail hypodermal cells, suggesting that LIN-44 signal can act over that distance. However, it is possible that the B and tail hypodermal cells are in direct contact through cellular projections or that the signal from the tail hypodermal cells is transmitted to the B cell by intervening cells.

Wnt proteins appear to act through an evolutionarily conserved signal transduction pathway (Klingensmith and Nusse, 1994). The best characterized Wnt signaling pathway is the *wg* pathway, which functions in the control of segment polarity in Drosophila. Analysis of other mutants with segment polarity defects have identified several potential components of wg signaling. Thus, it has been suggested that the product of *porcupine* is required for secretion of wg (Siegfried et al., 1994) and that the products of *armadillo* (Peifer and Wieschaus, 1990; McCrea et al., 1991), *dishevelled* (Klingensmith et al., 1994; Theisen et al., 1994), and *zeste-white 3* (Siegfried et al., 1992), along with an unknown wg receptor, act in the reception and transduction of the wg signal. Nematode homologs of these genes have not yet been reported.

Model for LIN-44 Function

We propose that LIN-44 functions as a signal from the tail hypodermis and controls the polarities of the B and T cells or their descendants (Figure 7). The polarities of the F and U cells are known to depend on the presence of the B cell (Chisholm and Hodgkin, 1989; Herman and Horvitz, 1994); furthermore, in *lin-44* mutants, the F and U cells exhibit polarity reversals if and only if the B cell undergoes polarity

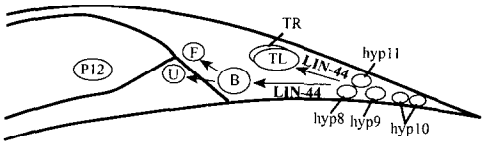

Figure 7. Model for LIN-44 Function

Schematic view of the tail of a newly hatched L1 animal showing nuclei only of cells that are affected by *lin-44* mutations or that appear to be involved in LIN-44 function. We propose that LIN-44 functions as a signal from the tail hypodermal cells hyp8, hyp9, hyp10, and hyp11 and that this signal instructs the B and T cells about their polarity. The LIN-44 signal may function to provide either directional information or a permissive environment.

reversal (Herman and Horvitz, 1994). We therefore suggest that the B cell transmits polarity information to the F and U cells after receiving LIN-44 signal from the tail hypodermis. (T cell polarity reversals are uncorrelated with the polarity reversals in B, F, and U.)

What could the LIN-44 signal be telling the B and T cells about their polarity? One possibility is that the LIN-44 signal provides directional information, for example, by marking as posterior the sides of the T and B cells that receive signal. Since the tail hypodermal cells are posterior to B and T, they are in a good position to provide such directional information. In the context of this hypothesis, it may seem surprising that the *lin-44* defects were rescued very efficiently by *lin-44(+)* expression from a heat shock construct, since one might expect such expression to result in the presentation of LIN-44 to all sides of the T and B cells. On the other hand, the signal induced by heat shock could still be directional if another component necessary for processing or secreting the LIN-44 signal were restricted to the tail hypodermis. Alternatively, the LIN-44 signal might be permissive: the B and T cells might have polarity information that is activated by LIN-44 signal. Such a permissive signal would not instruct cells about their polarity but rather would allow them to express an intrinsic orientation.

lin-44 and Possibly *wg* Control Cell Polarity as Well as Cell Fate

Wnt genes have for some time been implicated in cell fate determination and the control of cell proliferation (Nusse and Varmus, 1992). *lin-44* mutants appear to be defective in the specification of one cell fate, that of P12 (Herman and Horvitz, 1994). Most defects in *lin-44* mutants, however, appear to be in the polarities of cells or cell divisions rather than in cell proliferation or in the specification of cell fates.

Although the most widely studied functions of *Wnt* genes involve the regulation of cell fate and cell proliferation, recent work suggests that *wg*, like *lin-44*, may play a role in controlling cell polarity (Theisen et al., 1994). Many structures on the cuticular surface of Drosophila display a tissue polarity, characterized by arrays of polarized sensory bristles or hairs; for example, each cell on the wing produces a distally pointing hair, and bristles and hairs on the thorax and abdomen point posteriorly. It has been proposed (Adler, 1992; Krasnow and Adler, 1994) that this polarity is established by an intercellular signaling pathway that includes the product of *dishevelled* (*dsh*), among other genes. Mutations in *dsh* disrupt the polarities (but not the structures) of bristles, hairs, and ommatidia. As already noted, *dsh* is implicated in the *wg* signaling pathway. Furthermore, Theisen et al. (1994) have reported that *wg* mutants and *dsh/+*; *wg/+* double heterozygotes exhibit polarity defects similar to those seen with *dsh* mutants. Theisen et al. (1994) have therefore proposed that both *dsh* and *wg* play important roles in establishing cell polarity as well as cell identity. *lin-44* may thus not be unique among *Wnt* genes in controlling cell polarity.

Experimental Procedures

Strains and Alleles

Nematodes were cultured by standard techniques (Sulston and Hodgkin, 1988). The following mutations were used: LGI, *unc-29(e1072)*, *hDf7 I* (Howell and Rose, 1990); LGIII, *ncl-1(e1865)*, *unc-36(e251)*; LGV, *him-5(e1490)*.

Y48F5 DNA Isolation

Y48F5 YAC DNA was separated from yeast chromosome DNA by pulsed-field electrophoresis (Schwartz and Cantor, 1984) using a 0.7% agarose gel in 0.5× TBE (Sambrook et al., 1989). The DNA was isolated by electroelution overnight in 0.5× TBE at 50 V using an Isco Model 1750-100 Electrophoretic Sample Concentrator (Lincoln, NE).

Generation of YAC Derivatives

We isolated derivatives of Y48F5 specific to each cosmid gap (Figure 1A) using gap repair in yeast (Ma et al., 1987) based on methods described by Miller et al. (1993). We used standard yeast techniques to construct the strain MHY2 (*Y48F5, MATa ura3-52, trp1, leu2-5, 112, lys2-1, ade2-1, his3Δ200*). To generate substrates for gap repair, we cloned fragments from the cosmids flanking each gap into the centromere-containing *LEU2* YCplac111 vector (Gietz and Sugino, 1988). For SP#9, the left homologous fragment was a 2.7 kb HindIII–XbaI junction fragment from cosmid K08D2 cloned into the HindIII and XbaI sites in the multicloning site of YCplac111; the resulting clone was called SP#9L. The right homologous fragment was a 3.5 kb EcoRI fragment common to K05E6 and R12E2. This fragment, which has two internal XbaI sites, was cloned into the EcoRI site in the multicloning site of SP#9L; the resulting clone was called SP#9LR. SP#9LR was linearized by digestion with XbaI. The resulting molecule, which contained a yeast centromere and sequences flanking the cosmid gap at its ends, was transformed into the MHY2 strain (which contains Y48F5); leucine prototrophic transformants were selected. Out of 50 leucine prototrophic transformants tested, 47 were also prototrophic for tryptophan and uracil and therefore also contained Y48F5. These 47 transformants were grown in liquid media, selecting only for leucine prototrophy for approximately 20 generations to allow for spontaneous loss of the YAC, and then were streaked on plates lacking leucine and containing 5-flouro-orotic acid to select for Leu^+ Ura^- colonies. All 47 gave Leu^+ Ura^- colonies, which were then grown under selection for *LEU2*, transferred to nylon filters, and tested for hybridization with the gap flanking probes pMHS8 and pMHS9 (Figure 1B). Three yeast lines hybridized to both internal probes, but only one of the three was a tryptophan auxotroph, as expected if it no longer carried the YAC. We used an alkaline lysis procedure (Devenish and Newlon, 1982) to prepare SP#9 from this strain for microinjection experiments.

Genomic Library Construction and Screening

The pMHS9 probe (Figure 1B) was used to probe an unamplified λDASH II *C. elegans* genomic phage λ library provided by H. Browning and S. Strome. From approximately 20,000 plaques, we isolated two clones, SP#10 (data not shown) and SP#11 (Figure 1B). We constructed a genomic λ library from the yeast strain containing SP#9 in

the λDASH vector (Stratagene, La Jolla, CA) using the method of Kaiser and Murray (1985); we probed this library with pMHS8 (Figure 1B). From approximately 12,000 plaques plated on the CES200 (Wyman and Wertman, 1987) strain of E. coli, we isolated five clones, including SP#12 (Figure 1B).

Germline Transformation and *lin-44* Rescue

DNAs were microinjected into the mitotic germline of hermaphrodites according to the method of Mello et al. (1991). For rescue experiments, pRF4, a plasmid containing the semidominant *rol-6(su1006)* allele, was coinjected to identify transgenic (roller) lines. We scored each line for phasmid dye filling (Herman and Horvitz, 1994). A line was considered rescued if at least 50% of phasmids filled with dye.

Molecular Biology

Standard molecular biology methods (Sambrook et al., 1989) were followed, except where noted. All plasmid subcloning was done into pBluescript SK(−) (Stratagene, La Jolla, CA). Nested deletions of the 5.7 kb rescuing genomic fragment were prepared using the ExoIII–S1 method (Henikoff, 1987). A 4.1 kb deletion product rescued the *lin-44* phenotype in germline transformation experiments, and the DNA sequence of one strand of this clone was determined using Sequenase Version 2.0 (United States Biochemicals, Cleveland, OH) following the suggested protocol. Gaps in the sequence were closed by determining sequences using the following primers: GGATGGATTGACGATTGT, TCCCGACGATCAGTGGTG, CTCGTTTATGGTTTATGG, and GTATACACGTGTATAATTC. The 7.5 kb SalI–SacI rescuing fragment (Figure 1B) was used to probe a λZAP cDNA library provided by R. Barstead and R. Waterston. From approximately 120,000 plaques, we isolated eight cDNAs. Two of these cDNAs had identical restriction patterns and were shown to be specific to the 4.1 kb rescuing fragment. The DNA sequences of both strands of one of these clones were determined (Figure 2).

Identification of *lin-44* Mutations

We used PCR to clone genomic DNA from the *lin-44(n1792)* and *lin-44(n2111)* mutants. We used two sets of primers to amplify and clone the *lin-44* region in two halves with a small overlap between the halves. The primers used in the cloning were the following: 5′L, GAGAGCGGATAGACAGAGAG; 5′R, TATCTACAACTAAGGCCTCCG; 3′L, GAAGACATGCACGACACATC; 3′R, TGACACACATCATCCGATCC. The sequences of the regions corresponding to the exons were determined from at least three independent PCR clones for each allele. The primers used for sequence determination were the following: exon I, 5′L; exon II, TGCCTGCAGTCAGAAGCC; exon III, GTAGGTGTGGAACTTTTC; exon IV, GGATGGATTGACGATTGT; exon V, AAGTGACTTCAATGAAAC; exon VI, AGCCCCGTAAATCGACAC; exon VII, ATATTCTTGGGTCTCGCC.

Heat Shock Experiments

Plasmids containing the *lin-44* cDNA downstream of the *hsp16-2* and *hsp16-41* heat shock promoters (Stringham et al., 1992) were constructed. The presence of the control plasmids in transgenic lines was verified by PCR using the "reverse" sequencing primer from the vector and a primer in the *lin-44* coding region, GCTTCTCCACCTTCCTTAAC. To test whether the *hsp16::lin-44* cDNA fusion plasmids rescued *lin-44*, several hundred eggs were picked to a fresh plate. The plate was subjected to five 30 min heat shocks at 33°C separated by 90 min at 20°C. After the last heat shock, the animals remained at 20°C until they were scored for phasmid dye filling 3 days later.

Expression Experiments

pMHE1 is a fusion of the 837 bp XbaI–DraIII fragment immediately upstream of the *lin-44* coding region (Figure 1C) and the gene for GFP (Chalfie et al., 1994). pMHE3 is a fusion of the 2.6 kb fragment that extends from the end of the 7.5 kb genomic subclone to the DraIII site immediately upstream of the *lin-44* coding region (Figures 1B and 1C) and *lacZ* (Fire et al., 1990). pMHE4 is a fusion of the same 2.6 kb upstream fragment and the GFP gene. The cosmid C45D10 contains *unc-29(+)* (Lackner et al., 1994) and was used as a coinjectable marker for these experiments. Transgenic lines containing extrachromosomal arrays of these fusions and C45D10 were generated in the *unc-29(e1072)* background. *mnEx40* is a transgenic array containing pMHE3 and C45D10. We generated lines with integrated copies of *mnEx40* by γ irradiation (Mello and Fire, 1995). *mnIs9 X* and *mnIs10 X* are independent integrants of *mnEx40*.

β-Galactosidase expression in embryos was detected by staining fixed *unc-29; mnIs9* or *unc-29; mnIs10* embryos with anti-β-galactosidase antibodies (Promega, Madison, WI), diluted 1/250 with PBS (125 mM NaCl, 100 mM phosphate [pH 7.0–7.2]) for 2 hr at room temperature or at 4°C overnight and incubated with fluorescein-conjugated secondary antibodies for 2 hr at room temperature. The embryos were mounted for observation in 4 mM ascorbic acid, 0.01 μg/μl DAPI in PBS, and the coverslip was sealed with fingernail polish. β-galactosidase expression in larvae was detected using 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-Gal) staining (Fire et al., 1990). GFP expression in larvae was detected using an FITC filter set and epifluorescence microscopy.

In situ hybridization experiments were based on a modified method of Seydoux and Fire (1995) (H. Tabara and Y. Kohara, personal communication). Digoxygenin-labeled single-stranded DNA probes for hybridization were prepared as described by Seydoux and Fire (1995). The templates for both the antisense *lin-44* probe and the control sense *lin-44* probe were deletion derivatives of the *lin-44* cDNA that lacked the poly(A) tail. Probes were used at a concentration of 2.5–5 μg/ml in hybridization buffer (Seydoux and Fire,1995).

Mosaic Analysis

Animals of genotype *lin-44 I; ncl-1 unc-36 III* were made transgenic for an array containing wild-type copies of *lin-44* on the 7.5 kb genomic clone pMHS11, *ncl-1* on the cosmid C33C3, and *unc-36* on genomic clone R1p16 (obtained from L. Lobel). We estimated that the *mnEx31* array was lost at a frequency of approximately 0.01 to 0.02 per cell division.

The polarity of the T cell division can be assessed in L2, L3, and L4 animals by scoring the positions of the phasmid socket cells PHso1L/R (TL/R.paa) and PHso2L/R (TL/R.pap), which are anteriorly displaced in *lin-44* animals (Herman and Horvitz, 1994). B cell polarity was scored by the relative sizes of the daughter nuclei of the B cell division, B.a and B.p, in early-L2 males (Herman and Horvitz, 1994) or by the characteristic positions of the ten B cell descendants in late-L2 to mid-L3 males (Sulston and Horvitz, 1977).

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